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13. ABSTRACT ***(Maximum 200 Words)***

Although most of the tumors associated with neurofibromatosis type 1 (NF1) are benign in nature, malignant transformation of a subset of NF1 tumors is a serious complication, often leading to the death of the patient. This is true for NF1-associated juvenile chronic myelogenous leukemia (JCML), known to progress into acute myeloid leukemia (AML). Previously,we have shown that loss of *Nf1* in the hematopoetic lineage results in JCML. The goal of this grant is to identify the genetic events which lead to the transformation and leukemic progression of NF1-associated JCML using a mouse model. This model system takes advantage of transgenic mice that harbor one mutant allele of the *Nf1* gene, but require further mutations for transformation. We have backcrossed this mutant *Nf1* allele for three generations to a strain of mouse that expresses a murine leukemia virus (MuLV). In this system, the MuLV acts as a mutagen to activate cooperating cellular proto-oncogenes or inactivate tumor suppressor genes, resulting in accelerated tumor development. So far, by cloning sites of somatic MuLV integration from tumor DNAs obtained from these backcrossed mice, we have identified two genes that appear to cooperate with the loss of *Nf1* to cause to AML.

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Carrie Brannon 9/29/99

PI - Signature Date

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Introduction

Children with neurofibromatosis type 1 (NF1) have a markedly increased risk for juvenile chronic myelogenous leukemia (JCML) and monosomy 7 (Mo7) syndrome. Both JCML and Mo7 syndrome have a poor prognosis, with either progression to acute myeloid leukemia or death from incurrent problems. Using a mouse model of NF1, we demonstrated that the lack of the *Nf1* gene in hematopoietic cells is sufficient to cause chronic myeloid leukemia, but not acute myeloid leukemia, indicating that additional genetic events are responsible for the progression from chronic to acute disease. The goal of this proposal is to identify and isolate genes involved in this leukemic progression. Toward this end, we are using a mouse model system which takes advantage of mice that harbor one mutant allele of the *Nf1* gene but require further mutation for transformation to neoplasia. The general strategy is to breed the mutant *Nf1* allele onto a strain of mouse that expresses murine leukemia virus (MuLV) and exhibits a high incidence of acute myeloid leukemia. In this system, the MuLV acts as a somatic mutagen to activate cooperating cellular proto-oncogenes or inactivate tumor suppressor genes, resulting in accelerated tumor development. Since MuLVs activate proto-oncogenes by integrating nearby or inactivate tumor suppressor genes by integrating within the gene, the affected genes can thus be identified and cloned using these somatically acquired viruses as signposts. The most promising loci are the ones that have sustained proviral insertion in tumors of multiple mice. The identification of a so-called "common site of viral integration" strongly indicates that the region harbors a gene, that when mutated by the virus, is directly involved in the development of myeloid leukemia.

Technical Objective 1: Identify regions of the genome that cooperate with loss of *Nf1* in myeloid leukemia to cause tumor progression. Our objective is to produce a panel of 100 tumors derived from heterozygous N3 generation BXH-2 $Nf1^{Fcr}$/+ mice. This panel will then be used to identify new common sites of viral integration. This objective is divided into six tasks.

Progress on Task 1: Months 1-10: *Produce F_1, then N_2, followed by N_3 BXH-2 $Nf1^{Fcr}$/+ mice.*
Our initial strategy was to complete production of each generation before going onto the next. However, in practice, this strategy has not worked, mainly because the parental BXH-2 strain to which we are backcrossing has had small litters. Therefore, we have staggered the breeding to keep in line with the BXH-2 production. This has extended the time frame of this task, but the modified breeding scheme seems to be working out. This past year, we completed all the matings required to produce the first two generations (F_1 and N_2). Currently, we have nine remaining mating cages going to generate additional N_3 generation mice.

Progress on Task 2: Months 8-17: *Age N_3 generation BXH-2 $Nf1^{Fcr}$/+ mice.*
Presently, we have 33 animals in aging. These mice are being examined once per day to identify those animals that have developed acute myeloid leukemia and are ready to be sacrificed. We expect that additional N_3 mice will soon be added to this aging study upon weaning from the mating cages described in Task 1.

Progress on Task 3: Months 9-18: *Collect moribund animals. Process tumor sample. Assess the status of the wild-type Nf1 allele; determine the number of somatically acquired viral integrations; phenotypic analysis.*
To date, our panel consists of 52 N_3 BXH-2 $Nf1^{Fcr}$/+ tumors. Each of these tumors have been characterized for loss of the remaining normal *Nf1* allele and the number of somatically acquired viral integrations. In addition, we have 13 other tumors in the process of being characterized. Once this is done, we will have a total of 65 tumors, approximately two-thirds of the way toward our 100 tumor goal. With the number of N_2 animals currently in aging as well as those that will be born in the next several months, we anticipate that we will come close to reaching our 100 tumor goal.

Progress on Task 4: Months 1- 24: *Clone somatically acquired proviruses from tumor samples which harbor defects in the wild-type Nf1 allele and have only one or two somatic proviral insertions. Prepare genomic flanking probes to screen tumor panel.*
This year, we cloned 5 sites of viral integration. After the identification of non-repetitive flanking probes, we screened the tumor panel prepared in Task 5 and determined that one recognized a common site of viral integration (31% of tumors harbor a viral integration at this site, see Task 6 below).

Progress on Task 5: Months 18-19: *Prepare Southern blot containing the panel of tumor DNA samples which harbor defects in the wild-type Nf1 allele. Screen with probes to identify common sites of viral integration.*

The Southern blots have been prepared using all the available tumor samples. They have been screened an used to identify a common site of viral integration present in 31% of the tumors. Certainly, as more tumor samples are generated from the aging study in Tasks 2 and 3, we will be able to make additional Southern blots.

Progress on Task 6: Months 18-36: *Use probes that recognize common sites of viral insertion to isolate cosmid DNA. Begin studies to identify and characterize affected gene.*

After having identified a common site of viral integration, we screened a BAC (Bacterial Artifical Chromosome) library with the flanking probe rather than a cosmid library because BACs contain larger inserts of genomic DNA (especially after our experience on Technical Objective 2 - see below). In addition, we sent the flanking probe to our collaborators in Frederick, Maryland to map the sites on the mouse genome. They determined that our site mapped to chromosome 10 and was genetically linked to the *c-myb* gene. Using probes derived from the *c-myb* locus, we found that the two loci were contained on the BAC, indicating that they are also physically linked. Further analysis has revealed that the majority of integrations have occurred at the 3' end of *c-myb*. Table 1 lists the 16 tumors that have so far been identified as having a viral integration in or near *c-myb*. Interestingly, the majority of the viral integrations have occurred 3' of the *c-myb* gene, however there are a few which contain integrations within the gene. Table 1 also illustrates that while two of the tumors (#10 and #11) do not exhibit an obvious second hit at the *Nf1* locus (we cannot rule out an inactivating point mutation, for example), the other fourteen tumors have either LOH or an *Evi-2* integration. Furthermore, we found that three tumors (#14, #355 and #371) have only the single viral integration at the *c-myb* locus. Together, these data suggest that loss of *Nf1* function cooperating with deregulation of *c-myb* may be sufficient to induce acute leukemia. Currently, we are isolating more probes from the BAC clone to determine if we can detect any additional rearrangements in other tumors in the panel in the vicinity of *c-myb*.

Table 1. Tumors containing somatic viral integrations near *c-myb*

Animal Number	LOH	Evi-2	Number of Integrations	Myb Insertion	3' of Myb Insertion
7	+	-	4	+	-
8	+	-	2	+	-
10	-	-	3	-	+
11	-	-	1	-	+
13	+	-	3	+	+
14	-	+	1 + *Evi-2*	-	+
33	+	-	3	-	+
35	+	-	5	-	+
84	+	-	2	-	+
103	+	-	2	+	-
355	+	-	1	-	+
368	+	-	5	-	+
468					
371	+	-	1	-	+
419	+	-	2	-	+
522	+	-	5	-	+

In addition to identifying additional tumors containing *c-myb* integrations, we have started to characterize the tumors in Table 1 at the molecular level. We have analyzed the tumors by Southern blot and found that they have no detectable rearrangements of the T-cell receptor genes (TCRβ1 or TCRβ2), or the immunoglobulin heavy chain gene, indicating that the tumors are not lymphoid in origin. Using Northern blot analysis, we found that the tumors express the myeloid cell markers, myeloperoxidase and *c-fms*, further supporting the hypothesis that these tumors represent either an M2- or an M4-subtype acute myeloid leukemia. In the coming few months, after completing the phenotypic characterization of these tumors, we anticipate publishing these findings.

Finally, it is important to determine how these viral insertions affect *c-myb* expression. We have taken two approaches. First, we are examining the orientation and location of all these sites of viral

integration listed in Table 1 relative to the *c-myb* gene. If gene activation is occurring by an enhancer insertion mechanism, then we would predict that the viruses integrating 3' of *c-myb* will be in the same transcriptional orientation. Second, we have examined the levels of *c-myb* expression in the tumors using a Northern blot containing total RNA isolated from tumors in Table 1. Initially, we were disappointed to find that *c-myb* expression levels do not appear to be increased in tumors containing a viral insertion near the *c-myb* locus relative to those not containing a viral insertion near the *c-myb* locus (See Figure 1). However, after searching the literature, we found that the lack of overexpression of *c-myb* in tumors is not without precedent. Previously, Jiang *et. al* reported their experimental data resulting from infection of newborn mice with a combination of Abelson MuLV (contains the *v-abl* oncogene) and Moloney MuLV (an insertional mutagen) to induce B-cell lymphoma (Jiang et al., 1994). They determined that 16% of these tumors had Moloney MuLV insertions 3' of *c-myb*, but found that these tumors did not result in overexpression of *c-myb*. Based on these results, Jiang *et. al* concluded that the viral insertions activated an as yet unidentified gene. However, our interpretation of these data is that the presence of the viral *cis*-regulatory elements downstream of *c-myb* do affect *c-myb* expression, but not by upregulating transcription. Rather, we speculate that the virus serves to prevent *c-myb* downregulation, a process that appears to be essential for terminal differentiation of the myeloid and B-cell lineages (reviewed in Oh, 1999). If our hypothesis is correct, then we would predict that dysregulation of *c-myb* in combination with expression of *v-abl* induces B-cell lymphoma, while dysregulation of *c-myb* in combination with the loss of *Nf1* function causes acute myeloid leukemia.

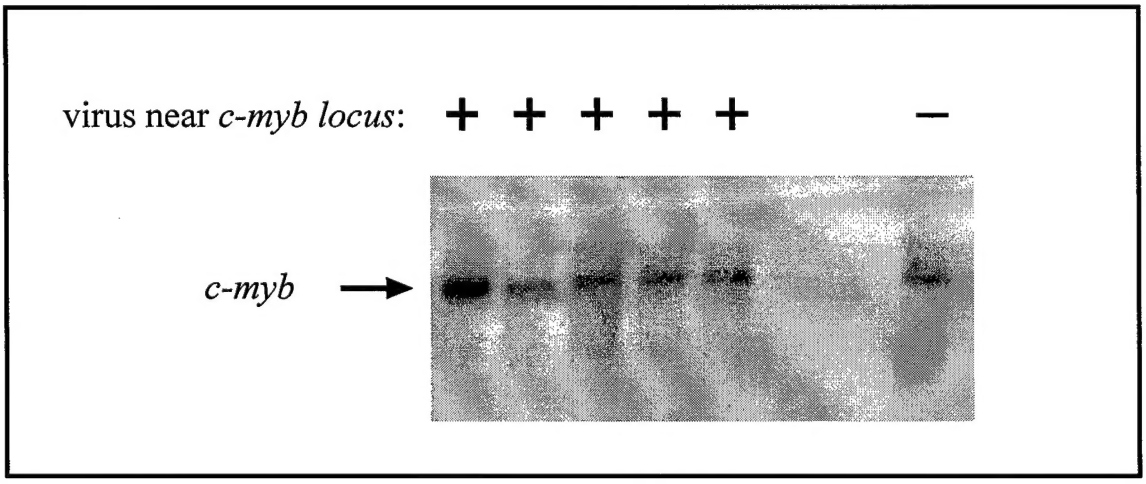

Figure 1. Northern blot of tumor RNA probed with a *c-myb* cDNA probe. Shown are five tumors containing (+) and one tumor lacking (-) a viral insertion near the *c-myb* locus.

Technical Objective 2: Characterize a locus containing a gene involved in tumor progression. Our objective is to identify the gene that is affected by a common site of viral integration. We have divided this objective into 4 tasks (note: we have combined the progress report for the first two tasks).

Progress on Tasks 1 and 2: Months 1-6: *Exon trap cosmid isolated with flanking probe. Determine if exons are candidate genes.*

Previously, we isolated a probe flanking the proviral integration in a tumor which we had shown lacked a normal copy of the *Nf1* gene. We showed that this flanking probe recognized a similar viral insertion in another tumor which had also sustained loss of *Nf1*. Together, these data indicated that we had identified a locus that upon insertional mutagenesis, was capable of cooperating with the loss of *Nf1* to cause acute myeloid leukemia. Therefore, we have sought to identify the gene affected by the proviral insertion. Initially, we began by isolating a 40 kb mouse genomic clone from a cosmid library using the flanking probe. We exon trapped this cosmid to identify putative exons in the vicinity of the proviral insertion (Church et al., 1994), but could not find any exons that we could show were transcribed. Therefore, since we were unable to convince ourselves that we have identified the gene affected by the proviral integration, and because the site of integration mapped very close to the end of the cosmid, we extended our analysis beyond this 40 kb region by obtaining a BAC clone. After our collaborators mapped the flanking probe to a region genetically linked to two candidate genes, we used the BAC clone to investigate whether either candidate gene was physically linked. We determined that neither was contained on the BAC, indicating that we had no viable candidate gene. Thus, we began sequencing the BAC clone to identify the gene affected by the common site of viral integration. Luckily, we found evidence of a putative first exon of a novel gene as it contained a start codon followed by an open reading frame and a splice donor signal. Furthermore, using RT-PCR, we have determined that this exon is transcribed in the tumor, in testes, and during embryonic development. We believe that this exon was most likely missed in our earlier exon trapping experiments due to the facts that only internal exons are captured by this technique and the site of integration mapped very close to the end of the cosmid.

Progress on Task 3: Months 7-18: *Isolate cDNA clones. Determine the genomic structure and normal expression pattern of the gene. Determine orientation of the gene relative to the viral integrations. Establish if an virus activates or eliminates gene expression.*

Due to the recent information derived from the sequence and expression analysis as described above, we now have obtained evidence that we have identified an exon of the affected gene. Therefore, in the coming year, we will be isolating cDNA clones to more fully characterize the gene. Once cDNA clones are isolated, they will be sequenced and compared to the genomic locus to determine exon-intron structure.

Finally, because we have evidence that the viral integrations appear to have occurred at the 5' end of gene, it is our hypothesis that the somatically acquired virus has resulted in activation of this novel gene. Northern analysis of tumor RNA is in progress to test this hypothesis.

Progress on Task 4: Months 13-36: *Create knock-out construct for the isolated gene. Transfect and isolate ES clones that have undergone gene targeting. Inject ES clones into blastocysts to generate a line of mice that harbor the gene knock-out mutation in their germline. Study phenotype of mice homozygous for the mutation.*

Due to the delay in identifying the affected gene, we have had to delay work on this precise task. However, while waiting, we have instead created a novel mutation in the *Nf1* gene (see below).

The *NF1* gene encodes neurofibromin, a large protein with multiple isoforms produced as a result of alternative splicing. One of these alternatively spliced exons is exon 23a which encodes 21 amino acids (aa) in the middle of the GAP-related domain. Type I neurofibromin has been defined as not the isoform lacking these additional 21 aa whereas the type II isoform contains them. Others have shown that type II neurofibromin has a 10-fold decrease in GTPase activating protein (GAP) activity, but has a greater affinity for *Ras* (Andersen et al., 1993). In additional, some have suggested that the ratio of type I to type II isoforms may reflect and/or influence differentiation status (Gutmann et al., 1993; Nishi et al., 1991). Therefore, to investigate the function of type II isoform of neurofibromin, we sought to generate mice that specifically lack exon 23a (*Nf123a*$^{-/-}$). First, we isolated genomic DNA surrounding exon 23a and constructed a targeting vector which replaces exon 23a with a selectable marker. After electroporation of the targeting vector into embryonic stem (ES) cells, and subsequent selection, we found that 1 out of every 9 clones contained the desired replacement of exon 23a. We then injected two independent clones into blastocycts and successfully obtained germline transmission of the exon 23a deletion mutation. So far, using intercrossing of heterozygous mice, we have been able to show that mice homozygous for this mutation are viable and lack type II isoform in brain tissue (Fig. 2). In the coming year, we propose to fully characterize the mutant mice at the histological and behavioral level.

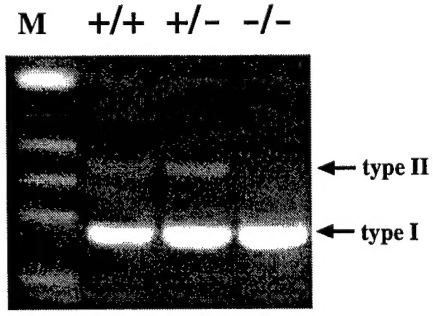

Figure 2. *Nf123a*$^{-/-}$ mice lack the Nf1 type II isoform. RT-PCR analysis of brain RNA using oligonucleotide primers in exons 23 and 24. Type I isoform is 271 bp, type II is 334 bp. M represents the molecular weight marker lane, followed by representative animals of all three genotypes.

Key Research Accomplishments

- Production of a panel of murine acute myeloid leukemia tumors that lack the wild type *Nf1* gene product
- Identification of a gene which appears to cooperate with the loss of *Nf1* to cause acute myeloid leukemia (affected in 31% of the tumors in panel)
- Preliminary identification of a gene which appears to cooperate with the loss of *Nf1* to cause acute myeloid leukemia (affected in 4% of the tumors in panel)
- Generation of a novel strain of mouse containing a deletion mutation of the alternatively spliced *Nf1*, exon 23a.

Reportable Outcomes

- An abstract presented at the National Neurofibromatosis Foundation meeting
- A repository of murine acute myeloid leukemia tumors
- Generation of a strain of mouse lacking the alternatively spliced *Nf1* exon 23a.
- Submission of a grant application to the Department of Defense based on the identification of two genes which appear to cooperate with mutations in the Nf1 gene to cause acute myeloid leukemia
- successful placement of a former postdoctoral fellow, Tao Yang, in a pathology residency program in Jacksonville, Fl

Conclusions

Our hypothesis has been that progression of juvenile chronic myelogenous leukemia (JCML) to acute leukemia (AML) is the result of genetic mutations in cooperating genes in addition to loss of *NF1*. In the two years of this study, we have successfully identified two genes that appear to be candidates for JCML to AML tumor progression. Therefore, this data strongly supports our hypothesis and has provided us with valuable reagents which we can use to experimentally prove our hypothesis.

So what does this research mean to NF1 patients? While most of the tumors associated with neurofibromatosis type 1 (NF1) are benign in nature, unfortunately, malignant transformation of a subset of NF1 tumors can be a serious complication. For example, plexiform neurofibromas are known to transform into malignant nerve sheath tumors (MNPSTs) and juvenile chronic myelogenous leukemia (JCML) is known to progress into acute leukemia (AML). Because of our research, we now have a handle on the types of genetic changes which lead to the formation, transformation and leukemic progression of NF1-associated JCML. This in turn may lead better prevention, diagnosis, and rational treatment for all malignancies that affect NF1 patients.

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